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cis Elements and *trans*-Acting Factors Involved in Dimer Formation of Murine Leukemia Virus RNA

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The genetic material of all retroviruses examined so far consists of two identical RNA molecules joined at their 5′ ends by the dimer linkage structure (DLS). Since the precise location of the DLS as well as the mechanism and role(s) of RNA dimerization remain unclear, we analyzed the dimerization process of Moloney murine leukemia virus (MoMuLV) genomic RNA. For this purpose we derived an in vitro model for RNA dimerization. By using this model, murine leukemia virus RNA was shown to form dimeric molecules. Deletion mutagenesis in the 620-nucleotide leader of MoMuLV RNA showed that the dimer promoting sequences are located within the encapsidation element *Psi* between positions 215 and 420. Furthermore, hybridization assays in which DNA oligomers were used to probe monomer and dimer forms of MoMuLV RNA indicated that the DLS probably maps between positions 280 and 330 from the RNA 5′ end. Also, retroviral nucleocapsid protein was shown to catalyze dimerization of MoMuLV RNA and to be tightly bound to genomic dimer RNA in virions. These results suggest that MoMuLV RNA dimerization and encapsidation are probably controlled by the same *cis* element, *Psi*, and *trans*-acting factor, nucleocapsid protein, and thus might be linked during virion formation.

Cells infected with Moloney murine leukemia virus (MoMuLV) produce three major size classes of virus-specific RNAs that are the 22S, 35S, and 70S RNAs (5). Most of the 22S and 35S RNA is found in free cytoplasm, while most of the 70S RNA is in the membrane-bound fraction (9). The MoMuLV 22S RNA is the messenger for the $Pr85^{env}$ precursor and is not found in virions. The 35S RNA is the genome-length RNA of 8,332 nucleotides (nt) which assumes two functions: it is the messenger RNA for the $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ polyproteins and the precursor to the genomic dimer 70S RNA found in mature virions (5). The preferential packaging of the genome-length murine leukemia virus (MuLV) RNA in virions is dependent upon a *cis* element, named *Psi*, first mapped between positions 215 to 565 from the RNA 5′ end (20, 21). Furthermore, it has been shown that only the upstream part of *Psi* between positions 215 to 400 is required for MoMuLV RNA packaging (33).

In the genomic RNA dimer, the two identical subunits are annealed to each other at the dimer linkage structure (DLS) located within the 5′-leader sequence of MuLV genome (2, 3, 28). The DLS holds together two identical RNA molecules by an apparent 5′ to 5′ linkage (15): such a conformation cannot result from direct intermolecular secondary interactions and must imply either other cofactors, as previously suggested (15), or tertiary structure motifs (39).

However, although the DLS has been identified by electron microscopy around position 400 from the MoMuLV RNA 5′ end (28), its precise location and the possible mechanism and function of RNA dimerization clearly remained to be determined. Thus, in an attempt to characterize the structure of the dimer linkage and to identify the mechanism of RNA dimerization, we initiated a study of the DLS and of RNA dimerization in vitro. MoMuLV RNA with the leader (620 nucleotides) and the 5′ *gag* sequences was synthesized in vitro, and its ability to form dimeric RNA molecules was monitored. In addition, we tried to define *cis* elements and *trans*-acting factors controlling MoMuLV RNA dimerization in vitro and we mapped the DLS by using complementary DNA oligonucleotides either to probe monomeric and dimeric RNA molecules or to block RNA dimerization.

The results reported here show that RNA dimerization occurs spontaneously, implying direct interactions between the two RNA molecules, and that it is catalyzed by nucleocapsid protein NCp10 (16). The sequences involved in the DLS and needed for RNA dimerization were mapped within the *Psi* encapsidation element (positions 215 to 400; see references 20, 21, and 33). Finally, a study of the RNA-protein interactions taking place in MoMuLV virions indicated that NCp10 is the major RNA-linked protein.

MATERIALS AND METHODS

Plasmid constructions. Standard procedures were used for restriction digestion and plasmid construction (19). *Escherichia coli* HB 101 (1035) was used for plasmid amplification. A diagram of these constructions is shown in Fig. 1.

(i) pMLVAC-7. The 4.9-kilobase (kb) *Sac*I-*Hin*dIII fragment (MuLV genome between positions −30 and 4894) of the pMLVK MoMuLV proviral DNA (25, 35) was subcloned into the pSP65 DNA vector. pMLVAC-7 contains the complete leader sequence plus the *gag* and part of the *pol* genes, under the control of the SP6 promoter for transcription.

(ii) pMLVAC-4. The 5.2-kb *Pst*I-*Sma*I fragment (positions 567 to 5750) of the pMLVK MoMuLV proviral DNA (25, 35) was subcloned into the pSP64 DNA vector, with a partial digest by the *Pst*I enzyme. pMLVAC-4 contains a leader sequence truncated up to position 567 (AUG codon at positions 621 to 623).

(iii) pMLVAC-5. The 5-kb *Pst*I-*Sma*I fragment (positions 743 to 5750) of the pMLVK DNA was subcloned into the

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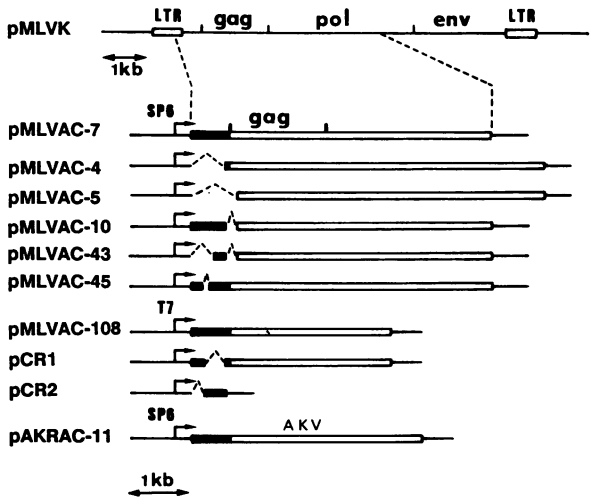

FIG. 1. Diagram of plasmid constructions. MoMuLV DNA fragments (represented by a box) from the proviral DNA pMLVK (25) were subcloned into pSP65, pSP64, or Blue Scribe DNA vectors. Deletions (broken lines) were made within the 620-nt leader sequence (solid box). All constructions are detailed in Materials and Methods. pAKRAC-11 contains an insert of AKV genome subcloned from pAKRAX-57 (14). Long terminal repeat (LTR), *gag*, *pol*, and *env* genes are indicated above in the pMLVK drawing. Nucleotide +1, referred to in the text, corresponds to the first nucleotide of MoMuLV RNA genome.

pSP64 DNA vector. In this plasmid the leader sequence has been completely removed.

(iv) pMLVAC-10. To remove the *gag* AUG, pMLVAC-7 was digested by *Pst*I, treated by T4 DNA polymerase, and ligated, resulting in a deletion from 567 to 747, with the upstream part in frame with the *gag* gene.

(v) pMLVAC-43. The pMLVAC-10 plasmid was digested with the *Eco*RI enzyme (site in the vector polylinker just upstream the *Sac*I site at position −30) and partially with the *Aat*II enzyme, to remove the 0.4-kb fragment located 5′ to the *Aat*II site (position 367). The plasmid was recircularized with *Eco*RI linkers.

(vi) pMLVAC-45. pMLVAC-7 was used to delete the sequence from 182 to 312. The deletion was carried out with a partial *Ava*II digestion (*Ava*II sites at 182 and 312) followed by ligation (30).

(vii) pMLVAC-108. The pMLVAC-7 *Eco*RI-*Bam*HI fragment (MoMuLV genome from positions −30 to 3225, *Eco*RI site just upstream *Sac*I site in pSP65 polylinker) was subcloned into the Blue Scribe vector (Genofit).

(viii) pCR1. The pMLVAC-108 *Sac*I fragment (−30 to 2558) was removed and replaced by the equivalent *Sac*I fragment from pMOV-3 with the *Psi* element 215 to 565 deleted (21).

(ix) pCR2. the pMLVK *Ball*-*Pst*I fragment, extending from position 215 to position 657, was inserted into the Blue Scribe vector treated by *Sac*I and Klenow enzymes (to generate blunt ends) and then by *Pst*I.

(x) pAKRAC-11. The pAKRAX-59 plasmid containing the AKR murine leukemia virus (AKV) genome (14) cloned in pBR 327 was used to subclone a 3.7-kb fragment, extending from the *Sma*I (position 27) to *Sal*I (position 3720) sites, into the pSP65 vector.

RNA in vitro synthesis, purification, and analysis. MoMuLV recombinant plasmids were linearized at the *Bst*EII site (position 725) or at the *Xho*I site (position 1560), except for the pMLVAC-5 plasmid, which was linearized at the *Pvu*II site (position 1436), and the pCR2 plasmid, which was linearized at the polylinker *Hin*dIII site (position 570) or at *Pvu*I site (position 421). The AKV recombinant plasmid was linearized at the *Bst*EII site (position 745). The linearized DNA (2 μg) was transcribed for 60 min at 37°C by 20 U of SP6 or T7 RNA polymerase (Promega Biotec) in 40 mM Tris hydrochloride (pH 8)–6 mM $MgCl_2$–2 mM spermidine–10 mM dithiothreitol–0.5 mM each ribonucleotide triphosphate–80 U of RNasin (final volume 0.1 ml). Following a 15-min DNase I treatment (2 U), the viral RNA preparation was extracted twice with phenol-chloroform, precipitated twice in 0.15 M sodium acetate with 3 volumes of ethanol, and dissolved in sterile bidistilled H_2O. ^{32}P-labeled RNA (Fig. 5D) was synthesized in the same conditions, except that 10 μCi of [α-^{32}P]UTP was added and UTP concentration was lowered to 30 μM. RNA (0.5 μg) was analyzed by native 0.8% agarose gel electrophoresis in 50 mM Tris borate–0.1 mM EDTA, pH 8.3, at 7 V/cm, followed by staining with ethidium bromide at 1 μg/ml for 5 min.

Purification of MuLV NCp10 and RSV NCp12 for dimerization assays. Purification of MuLV NCp10 and Rous sarcoma virus (RSV) NCp12 was carried out as follows: MuLV-producing 3T3-A9 cells (10) and RSV-infected chick embryo fibroblasts were grown in Dulbecco modified medium containing 5% fetal calf serum. To purify large amounts of virions, cells were grown in bottles and medium was collected every 12 h. Virus was prepared according to published procedures (7, 8). Starting from 50 mg of virions, MuLV-NCp10 and RSV-NCp12 were purified by DNA-cellulose chromatography by the protocol described by Moelling et al. (26). Both proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by Coomassie blue staining. According to this analysis, NCp10 and NCp12 appeared pure.

RNA dimerization assay in vitro. RNA 0.5 μg was denatured in H_2O at 95°C for 1 min and incubated at 37°C for 10 min with 10 to 100 ng of MuLV NCp10 or RSV NCp12 and 5 U of RNase inhibitor RNasin in 10 μl of dimerization buffer, with components (50 mM Tris hydrochloride [pH 7.0], 50 mM NaCl, 5 mM dithiothreitol, 10 μM $ZnCl_2$) selected for an optimized activity of NC protein (31). For RNA analysis, the protein was removed by phenol-SDS extraction before electrophoresis on a native agarose gel. The gel was washed in bidistilled water to remove phenol-SDS, and the RNA was stained as described above.

The DNA ^{32}P-labeled oligonucleotide annealing (Fig. 5B and C) was performed by adding DNA oligomers to RNA at the beginning of the dimerization reaction. The agarose gel was first stained with ethidium bromide to analyze the unlabeled RNA; it was then fixed in 5% trichloroacetic acid, dried down, and autoradiographed for 6 to 12 h at −80°C to analyze the oligonucleotide annealing. For the dimerization assays with ^{32}P-labeled RNA (Fig. 5D), the agarose gel was directly fixed in 5% trichloroacetic acid, dried down, and autoradiographed.

DNA oligomers. Synthetic oligomers were made in the laboratory and were complementary to MoMuLV RNA at positions 146 to 164, 260 to 279, 280 to 307, 310 to 329, and 378 to 395 for oligomers A, B, C, D, and E, respectively (see Fig. 5). They were purified by 8% polyacrylamide gel electrophoresis, 5′-end labeled with [γ-^{32}P]ATP by using T4 kinase, and purified by Sephadex G-50 filtration. The specific radioactivity obtained was between 10^7 to 10^8 cpm/μg of oligomer.

Electron microscopy. The 0.8-kb MoMuLV RNA (exact

length, 775 nt) was synthesized from pMLVAC-7 DNA as described above. RNA was purified on a Sephadex G-75 column (Pharmacia, Inc.) in 5 mM Tris, pH 7.5. 18S and 28S rRNAs were used as internal standards and gave the expected sizes.

RNA in 50 mM triethanolamine, pH 7.6, was partially denatured by dilution with an equal volume of 5 M urea and 100% formamide and incubation at 20°C for 1 h. RNA was spread by following the protein-free spreading procedure of Vollenweider et al. (41). Two volumes of a 2.5×10^{-3}% benzyldimethylalkylammonium chloride solution in 1.3% formamide were added to 1 volume of the partially denatured RNA solution at 600 ng/ml. A 50-μl drop of the mixture was placed onto a Parafilm sheet for 10 min, and the RNA film was picked up on a 600-mesh copper grid covered with a carbon film. The grid was quickly rinsed in water and stained in a solution containing 1 mM uranyl acetate, 1 mM HCl, and 90% ethanol for 30 s; it was then rinsed in 90% ethanol and rotary shadowed at an angle of 7°C by Platinium-Palladium. Electron micrographs were taken at magnification, ×50,000, at 60 kV on a JEOL 1200 EX.

Protein-RNA interactions in virions. UV cross-linking experiments with MoMuLV virions were carried out exactly as described previously with RSV (7), with exposition to a 252-nm UV light at 4°C and for 3 min. Virions were dissociated with 1% SDS–50 mM Tris hydrochloride [pH 7]–10 mM EDTA–0.15 M NaCl. The 70S RNA was purified by sucrose gradient centrifugation, extensively digested with T1 RNase, and immunoprecipitated with anti-MuLV serum on protein A-Sepharose beads (Pharmacia). The washed immunoprecipitate was treated with T4 kinase and [γ-^{32}P]ATP to label the cross-linked RNA oligonucleotide (which resulted in labeling the RNA-protein complex [22]). Then the protein was analyzed by SDS-PAGE electrophoresis on a 12.5% polyacrylamide gel (see Fig. 6). After electrophoresis, the gel was fixed with 10% acetic acid–30% methanol, dried, and autoradiographed for 16 h at −80°C. Viral proteins used as competitors were purified from virions by SDS-PAGE or acid-urea PAGE (for the basic NC protein), followed by electroelution from the gel at 120 mA for 3 h in 25 mM sodium acetate, pH 7.

Computer folding. The RNA 2 program of Zuker and Stiegler (42) was used. The RNA leader sequence of murine and rat viruses (38) was folded either without or with restrictions, such as sequences non-base-paired because of their function: the primer binding site (PBS) hybridized to primer tRNA, the splicing donor site involved in a loop structure (27) as well as initiator AUG (11). Furthermore partial T_1 RNase digestion and/or partial sequencing with avian myeloblastosis virus DNA polymerase gave additional information about the structure of MuLV leader RNA (A. C. Prats, Ph.D. thesis, Université Paul Sabatier, Toulouse, France, 1988).

RESULTS

Spontaneous dimerization of MoMuLV and AKV RNAs in vitro. In order to analyze dimerization of viral RNA in vitro, SP6-derived MoMuLV and AKV RNAs having the 620-nt leader and 5′ *gag* gene sequences were synthesized (Fig. 2A). The 0.8- and 1.6-kb RNAs, containing 105 and 940 nucleotides of 5′ *gag* sequences, respectively, were synthesized in a low-ionic-strength buffer (40 mM Tris, pH 8, 6 mM $MgCl_2$), dissolved in H_2O after purification and precipitation (see Materials and Methods), and analyzed by agarose gel electrophoresis before or after denaturation for 1 min at 95°C (Fig. 2B). Native MoMuLV and AKV RNAs appeared as two bands (lanes 1 and 3). The fast-migrating band had the expected molecular weight, whereas the other one corresponded to a dimer size RNA and disappeared upon heat denaturation for 1 min at 95°C (lanes 2 and 4). Dimerization could be restored after heat denaturation by incubating MuLV RNA in 0.1 M NaCl (lane 7). This spontaneous dimerization seemed specific to retroviral RNA, since neither ribosomal RNAs nor brome mosaic virus RNA gave rise to any dimeric RNA molecules (not shown). More precisely, it was specific to the 5′ end of MuLV genome, since a MoMuLV 0.7-kb RNA with the *gag* sequence from position 743 to position 1436 did not dimerize (Fig. 2B, lanes 5 and 6).

The requirement for divalent cations was assayed by incubating heat-denatured MoMuLV RNA in 0.1 M NaCl with EDTA or $MgCl_2$. As shown in Fig. 2B (lanes 7 through 9), addition of EDTA or $MgCl_2$ did not alter the rate of spontaneous dimerization. However spontaneous dimerization did not occur without NaCl and was less efficient at lower RNA concentrations (not shown; a detailed study of parameters governing RNA dimerization is in progress).

The denaturation of MoMuLV dimeric RNA generated by in vitro transcription was monitored and shown to occur between 50 and 60°C in 0.1 M NaCl (Fig. 2B, lanes 10 through 12), a temperature very close to the T_m of 55 to 60°C measured for MoMuLV 70S RNA (17).

In order to check that the putative dimeric RNA corresponds to a dimer and not to another conformation of the monomer, SP6-derived RNA was examined in the electron microscope after partial denaturation with 50% formamide and 2.5 M urea (see Materials and Methods). Figure 2C shows that the 0.8-kb RNA can be monomeric or dimeric. For all the dimers observed, the measure gave a length of 750 ± 50 nucleotides for each monomer, and the DLS at about 300 nucleotides from one end, presumably the 5′ end (see legend of Fig. 2C). Furthermore the estimated proportion of dimeric versus monomeric MoMuLV RNA seen by electron microscopy correlated well with the one observed by agarose gel electrophoresis.

Nucleocapsid protein catalyzes MuLV RNA dimerization. It has been noted in a previous report that nucleocapsid protein (NC), while activating the annealing of the replication primer $tRNA^{Pro}$ to the PBS of MuLV RNA (31), was also capable of causing MuLV RNA dimerization. This prompted us to analyze dimerization of MoMuLV RNA in the absence or presence of NC protein.

In order to monitor spontaneous dimerization, SP6-derived RNA was denatured and incubated at 37°C in a buffer optimized for NC protein activity (31), containing 50 mM NaCl, 50 mM Tris hydrochloride [pH 7], 5 mM dithiothreitol, and 10 μM $ZnCl_2$. As shown (Fig. 3, lanes 1 through 3) RNA dimerization occurred, but only about 80% of the RNA was in the dimer form after 3.5 h of incubation.

When MoMuLV RNA was incubated with NC protein, it was deproteinized before gel electrophoresis to prevent any gel retardation effect due to bound NC protein. The addition of NCp10 catalyzed RNA dimerization (Fig. 3, lanes 4 through 6), at a degree depending on the amount of protein added: RNA was about 50% dimerized in 10 min, with an estimated molar ratio of NC to RNA of 1.5 to 1 (lane 5), and was completely dimerized when the molar ratio was increased to about 5 (lane 6). An NC protein of heterologous origin, the NCp12 of RSV, was also able to activate dimerization of MuLV RNA (Fig. 3, lanes 7 through 9).

Sequences required and sufficient for MoMuLV RNA dimerization. In an attempt to identify the sequences re-

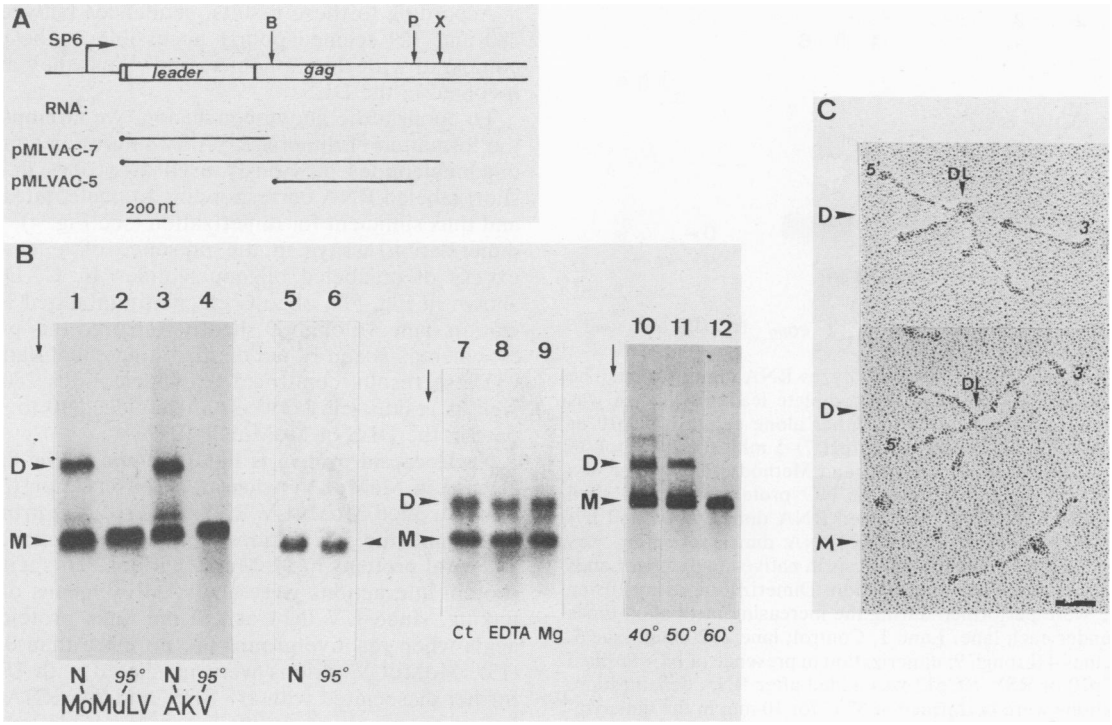

FIG. 2. (A) Diagram of MoMuLV RNAs tested for dimerization. MoMuLV leader and *gag* sequences were subcloned into an SP6 vector (Fig. 1). pMLVAC-7 DNA, containing the MoMuLV leader and *gag* and *pol* sequences, was cut by the *Bst*EII enzyme (B) at position 725 or by *Xho*I enzyme (X) at position 1560, leading to synthesis of two RNAs measuring 775 and 1,610 nt, respectively. pMLVAC-5 DNA contains only *gag* and *pol* sequences; the insert starts at position 743 of MuLV genome, whereas the leader has a length of 621 nt. pMLVAC-5 was cut by *Pvu*II enzyme (P) at position 1436, leading to synthesis of a 713-nt-length RNA. pAKRAC-11 DNA (not shown here) contains an AKV insert, with the leader and the *gag* gene homologous to the MoMuLV insert of pMLVAC-7. (B) Agarose gel electrophoresis of SP6-derived MuLV RNA dimers. Transcription of pMLVAC-7 (MoMuLV), pMLVAC-5 (MoMuLV without leader), and pAKRAC-11 (AKV) plasmids with SP6 RNA polymerase was carried out in the transcription buffer containing 40 mM Tris, pH 8, 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM dithiothreitol. RNA purification was done as described in Materials and Methods. The resulting RNAs (dissolved in H_2O after ethanol precipitation) were analyzed by native agarose gel electrophoresis, followed by ethidium bromide staining (see Materials and Methods.) Lanes 1 through 6, MoMuLV and AKV RNAs (0.5 μg), either native or after heating at 95°C for 1 min, were analyzed. Lane 1, 0.8 kb of native pMLVAC-7 RNA (MoMuLV); lane 2, 0.8 kb of denatured pMLVAC-7 RNA (MoMuLV); lane 3, 0.8 kb of native pAKRAC-11 RNA (AKV); lane 4, 0.8 kb of denatured pAKRAC-11 RNA (AKV); lane 5, 0.7 kb of native pMLVAC-5 RNA (no leader); lane 6, 0.7 kb of denatured pMLVAC-5 RNA (no leader). Note that 30 to 40% of the RNA is in the dimer form for the leader-containing RNAs. Lanes 7 through 9, 0.8 kb of pMLVAC-7 RNA was heated at 95°C for 1 min and then incubated at 37°C for 1 h in 100 mM NaCl–10 mM Tris, pH 8, with or without addition of 1 mM EDTA or 5 mM $MgCl_2$. Lane 7, NaCl; lane 8, plus EDTA; lane 9, plus $MgCl_2$. Lanes 10 through 12: 1.6 kb of pMLVAC-7 RNA (native form as in lane 1, 3, and 5) was incubated in 100 mM NaCl–10 mM Tris, pH 8, for 10 min at various temperatures. Heating temperatures: lane 10, 40°C; lane 11, 50°C; lane 12, 60°C (indicated under each lane). M and D indicate monomer and dimer RNA; the arrow is the direction of electrophoresis. (C) Electron microscopy of MuLV dimer RNA synthesized in vitro. The 0.8-kb MoMuLV RNA was spread as described in Materials and Methods. Among the molecules unwound enough to identify the RNA strands, there were monomers and dimers. Some dimers had other interstrand contact points, but one interaction at about 300 nt from one end was found in all understandable structures and identified as the DLS. Multimers and completely condensed molecules were seen, but their structures could not be interpreted. Two dimers (D) and one monomer (M) are shown at a final magnification of ×106,000. DL stands for dimer linkage structure. RNA 5′ and 3′ ends cannot be identified here; however, similar electron microscopy visualizations of the 1.6-kb RNA (A. C. Prats, Ph.D. thesis, Université Paul Sabatier, Toulouse, France, 1988) also showed a DLS at 300 nt from one end. Since the 0.8- and the 1.6-kb RNAs had the same 5′ end and since the 3′ part of the 1.6 kb RNA was unable to dimerize (Fig. 2B, lane 5), we concluded, in agreement with the mapping of the DLS shown in Fig. 5, that the 5′ end was at 300 nt from the DLS. Thus we have indicated the most probable 5′ and 3′ ends of RNA. The bar represents 150 bases.

quired for dimerization of MoMuLV RNA in vitro, deletions were made in the 620-nt leader of MoMuLV RNA (Fig. 4A), and the SP6- or T7-derived recombinant RNAs were analyzed by agarose gel electrophoresis to estimate their ability to dimerize (Fig. 4B).

Clearly the removal of the 5′ part of the leader (upstream from position 567 or 367) abolished RNA dimerization (Fig. 4B, lane 2 and 4), whereas deletion of the 3′ leader and of the 5′ *gag* sequences did not (lane 3). More precisely, dimerization was abolished by deletion of the *Psi* element (sequence 215 to 565 [20, 21]) or of a smaller sequence between positions 182 and 312 (Fig. 4A and B; RNA 5 and 6). On the other hand, RNA molecules composed of the *Psi* domain alone (sequence 215 to 565 as well as sequence 215 to 421 corresponding to the shortened *Psi* [20, 33]) were able to give dimers (RNA 7). Incubation of the deleted RNAs unable to dimerize with NC protein still did not lead to RNA dimerization (Fig. 4B, lanes 5 and 6).

These results indicate that residues between positions 215 and 312 and from 215 to 421 (the *Psi* encapsidation element), respectively, are required and sufficient to promote MoMuLV RNA dimerization in vitro.

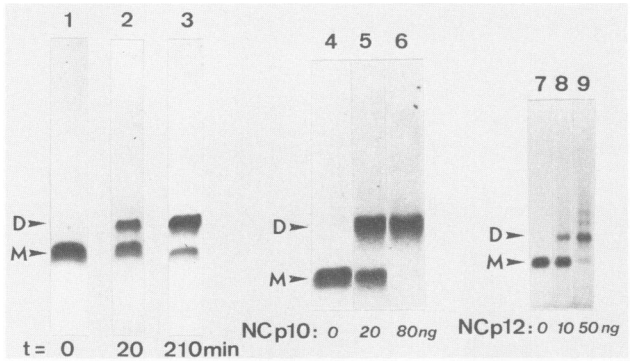

FIG. 3. Nucleocapsid protein catalyzes RNA dimerization. The 0.8-kb MoMuLV RNA having the complete leader sequence was denatured and incubated at 37°C, either alone or with NCp10 or NCp12 in 50 mM NaCl–50 mM Tris(pH 7)–5 mM dithiothreitol–10 μM $ZnCl_2$ as described in Materials and Methods. This buffer was selected for an optimal activity of NC protein, since we had observed that adding $ZnCl_2$ improved RNA dimerization and hybridization of primer $tRNA^{Pro}$ (31). RNA dimer formation was analyzed by agarose gel electrophoresis in native conditions. Lanes 1 through 3, Spontaneous dimerization. Dimerization assays using RNA alone were performed during the increasing incubation times indicated under each lane. Lane 1, Control; lane 2, 20 min; lane 3, 210 min. Lanes 4 through 9: dimerization in presence of NC. Purified MuLV NCp10 or RSV NCp12 was added after RNA denaturation, and incubations were performed at 37°C for 10 min in the dimerization buffer. RNA was deproteinized before electrophoresis (see Materials and Methods. Lane 4, Control without NCp12; lane 5, plus 20 ng of NCp10; lane 6, plus 80 ng NCp10; lane 7, Control without NCp12; lane 8, plus 10 ng of NCp12; lane 9, plus 50 ng NCp12. Amount of protein added is indicated under each lane. M and D are monomer and dimer RNA.

Mapping of the DLS of MoMuLV RNA by using complementary DNA oligomers. MoMuLV RNA sequences involved in the DLS were tentatively located by hybridizing to SP6-derived RNA a set of synthetic DNA oligomers complementary to domains of the leader sequence (Fig. 5A). The various 5′-^{32}P-labeled DNA oligomers were annealed to MoMuLV RNA in the presence of NCp10 or NCp12, and the hybridization level of each DNA oligomer to monomeric versus dimeric RNA was analyzed (Fig. 5B and C).

In a first series of assays, two DNA oligomers A and C, complementary to positions 146 to 164 (PBS) and 280 to 307 of MoMuLV RNA, respectively, were used (Fig. 5A). DNA oligomer A hybridized to both the monomeric and the dimeric RNA (Fig. 5B, lanes 2 and 3), whereas oligomer C hybridized only to the monomeric RNA (lanes 4 through 6). Annealing of these two oligomers was enhanced by NCp10 (lanes 3, 5, and 6), in agreement with our previous results (31). Figure 5C shows a second series of assays with four different DNA oligonucleotides, B, C, D, and E, complementary to positions 260 to 279, 280 to 307, 310 to 329, and 378 to 395, respectively (see Fig. 5A). First, the hybridization assays were performed with monomeric RNA alone, and we observed that oligomers B and C efficiently annealed to MoMuLV RNA (lanes 1 and 2), whereas oligomers D and E did not (lanes 3 and 4). In order to estimate the hybridization levels of these DNA oligomers to the monomer and dimer forms of MoMuLV RNA in the same reaction, NC protein was added so that about 60% of RNA was dimeric (Fig. 5C, lanes 5′ through 8′). As shown in lanes 5 through 8, DNA oligomers C and D mostly hybridized to the monomeric RNA (lanes 6 and 7), while oligomers B and E hybridized to both forms of MoMuLV RNA (lanes 5 and 8). According to these results, sequences between positions 280 and 329 seemed poorly accessible in the dimer RNA compared with the monomer, and, thus, they are probably involved in the DLS.

To confirm the above conclusion, we attempted to block the formation of dimeric RNA by saturating amounts of the oligonucleotides previously used as probes in Fig. 5C. A short labeled RNA corresponding to nucleotides 215 to 421, and thus sufficient for dimerization (see Fig. 4), was used in dimerization assays in the presence of a 50- to 100-fold excess of unlabeled oligonucleotides B, C, D, or E. As shown in Fig. 5D, oligo C efficiently inhibited RNA dimerization (lane 4); oligo D slightly impaired this process (lane 5), whereas B and E had no inhibiting effect (lanes 3 and 6).

These results confirmed that nucleotides 280 to 308, as well as residues in between nucleotides 310 to 329, participate in the DLS of MoMuLV RNA.

Nucleocapsid protein is tightly bound to the dimeric RNA genome in MoMuLV virions. The observation that NC protein activated MoMuLV RNA dimerization prompted us to reexamine the interactions between genomic dimer RNA and viral proteins in MoMuLV virions. The genomic RNA-protein interactions were analyzed by means of UV cross-linking, since UV light at 252 nm links protein to nucleic acids when reactive groups are no more than 0.1 nm apart (13). MoMuLV virions were irradiated with UV light and further dissociated with 1% SDS, and 70S RNA with cross-linked proteins was purified (see Materials and Methods). After RNase digestion, the proteins cross-linked to the genomic dimer RNA were identified by means of 5′ ^{32}P labeling, followed by immunoprecipitation with antiMuLV serum (see Materials and Methods). Results of the immunoprecipitation assays (Fig. 6) led to the identification of a major RNA-linked protein migrating around 10 kilodaltons and a minor one around 30 kilodaltons (lane 1). The major RNA-linked protein was identified by performing immunoprecipitation assays in the presence of an excess of the different *gag*-coded proteins. When NCp10 was the competitor, the major RNA-linked protein completely disappeared (Fig. 6, lane 2), whereas matrix protein MAp15 and phosphoprotein pp12 as competitors did not produce any significant loss (lane 3). Competitor capsid protein CAp30 removed only the 30-kilodalton migrating product (lane 4). The same results were obtained (not shown) with a chemical cross-linking agent, trans-diamine dichloroplatinum, that links protein to RNA when reactive groups are 0.7 nm apart (37). Thus, the major viral protein tightly bound to genomic dimer RNA in MoMuLV virions is the nucleocapsid protein NCp10 and not phosphoprotein pp12 as previously reported (34).

DISCUSSION

This report describes an in vitro system set up to study the dimerization process of MuLV RNA and the DLS holding together two identical RNA molecules. In vitro-generated MuLV RNA had the ability to spontaneously dimerize (Fig. 2), and this feature indicated that the DLS must correspond to direct interactions between the two RNA molecules, not a process involving any RNA or protein linker as previously suggested (15). Furthermore we showed that RNA dimerization in vitro was dependent upon *cis*-acting elements and was activated by the nucleocapsid protein (Fig. 3, 4, and 5).

The results obtained by using complementary oligonucleotide probes (Fig. 5) indicate that the DLS presumably maps at positions 280 to 330 from the 5′ end of MoMuLV RNA, in

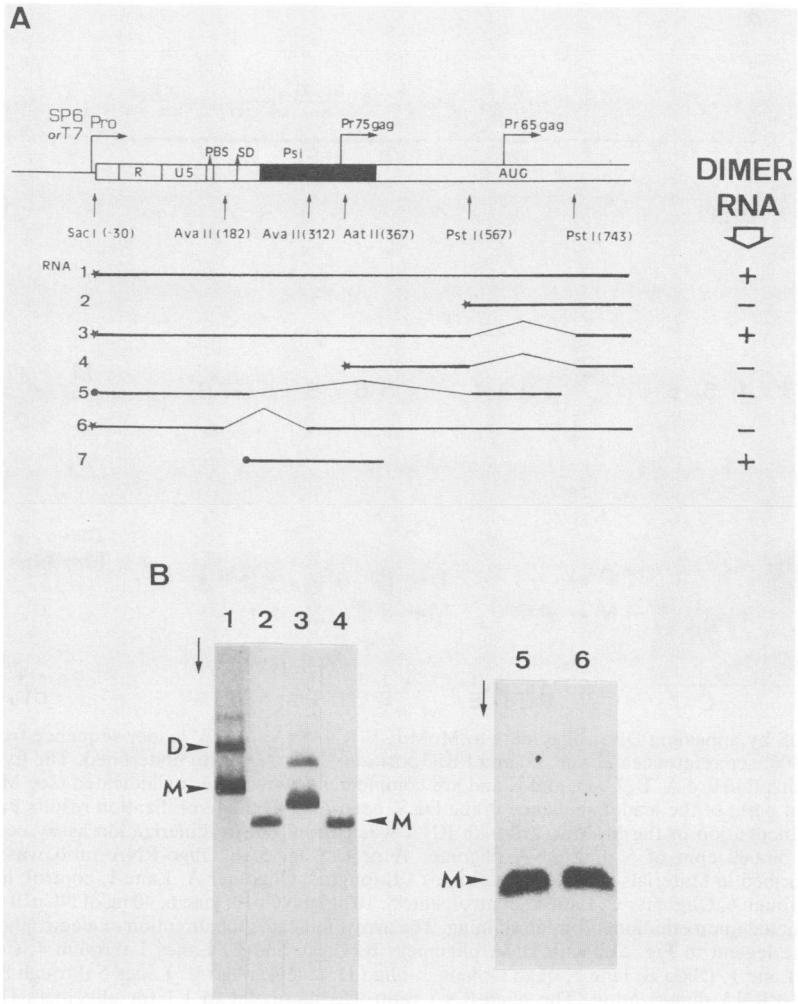

FIG. 4. Mapping of the sequences needed for MoMuLV RNA dimerization by deletion mutagenesis. (A) MoMuLV DNAs with different deletions in the leader sequence and linearized by different enzymes were used for transcription with SP6 or T7 RNA polymerase (see Materials and Methods). The MoMuLV 620 nucleotide leader is schematized. R, Terminal repeat; U5, 5′ untranslated sequence; PBS, primer $tRNA^{Pro}$ binding site; SD, splicing donor site at position 206; *Psi*, *cis* encapsidation element limited to nts 215 through 400 (21, 33). $Pr75^{gag}$ (precursor to the glycosylated *gag* protein) and $Pr65^{gag}$ translation initiation sites, localized at positions 357 (30) and 621, respectively, are indicated by arrows. Several restriction sites are shown: *Sac*I (position −30), *Ava*II (positions 182 and 312), *Aat*II (position 367), *Pst*I (positions 567 and 743) (38). Below the scheme of the leader are drawn the various recombinant MuLV RNAs. The 5′ ends are indicated by a star (SP6 RNA) or a full circle (T7 RNA), and deletions are shown by broken lines. The size of each RNA species used in the experiments is indicated. Lanes: 1, pMLVAC-7 RNA (0.8 or 1.6 kb): complete leader; 2, pMLVAC-4 RNA (1 kb): 5′ first 567 nts deleted; 3, pMLVAC-10 RNA (1.4 kb): nts 567 to 747 deleted; 4, pMLVAC-43 RNA (1 kb): 5′ first 367 nts and nts 567 to 747 deleted; 5, pCR1 RNA (0.4 kb): *Psi* deleted (nts 215 to 565); 6, pMLVAC-45 RNA (0.7 kb or 1.5 kb): nts 182-312 deleted; 7, pCR2 RNA (0.35 kb or 0.22 kb): contained *Psi* element, with either nts 215 to 565 or nts 215 to 421 (corresponding to shortened *Psi* [33]). The diagram represents the shorter 0.22 kb RNA. Dimerization assays were performed with these RNAs as described in the legends to Fig. 2 and 3. Results are summarized on the right for each RNA. Formation of dimer is indicated by +, absence of dimerization by −. (B) Some of the dimerization assays performed with deleted RNAs (Fig. 4A) are shown. Agarose gel electrophoresis and ethidium bromide staining were as described in Materials and Methods. Lanes 1 through 4, Native RNAs 1 through 4. Lane 1, 1.6 kb of RNA 1 (wild type); lane 2, 1 kb of RNA 2 (deletion from 5′ end to position 567); lane 3, 1.4 kb of RNA 3 (deletion 567 to 747); lane 4, 1 kb of RNA 4 (deletion from 5′ end to 367 and from 567 to 747). Lanes 5 and 6, Dimerization assay of RNA 6 (deletion 182 to 312) with NCp10 (see Fig. 2). Lane 5, ct; lane 6, plus 80 ng NCp10. M and D indicate monomer and dimer forms.

agreement with electron microscopy data of the MoMuLV 70S RNA carrying the primer $tRNA^{Pro}$ (2, 28). One might expect that the nucleotides 280 to 330 exhibit peculiar features justifying their direct participation in the DLS. In support of this notion, partial digestions of MoMuLV RNA with T1 RNase and computer folding analyses of the 620-nt leader of MoMuLV RNA show that nucleotides 280 to 330 fold into a double hairpin structure in the monomeric RNA (Fig. 7). The first hairpin (sequence 281 to 305) is accessible to oligo probe C (Fig. 5C) and is less stable than the second one (sequence 310 to 354) containing 30 GC residues with 12 GC pairs and poorly accessible to oligomer D (Fig. 5C). Computer folding analyses of the leader sequence of other retroviral RNAs show that this RNA secondary structure is conserved in seven other murine retroviruses (AKV, Friend MuLV, spleen focus-forming virus, Abelson murine leukemia virus, FBR-murine osteogenic sarcoma virus, replication-defective transforming murine sarcoma virus, radiation-

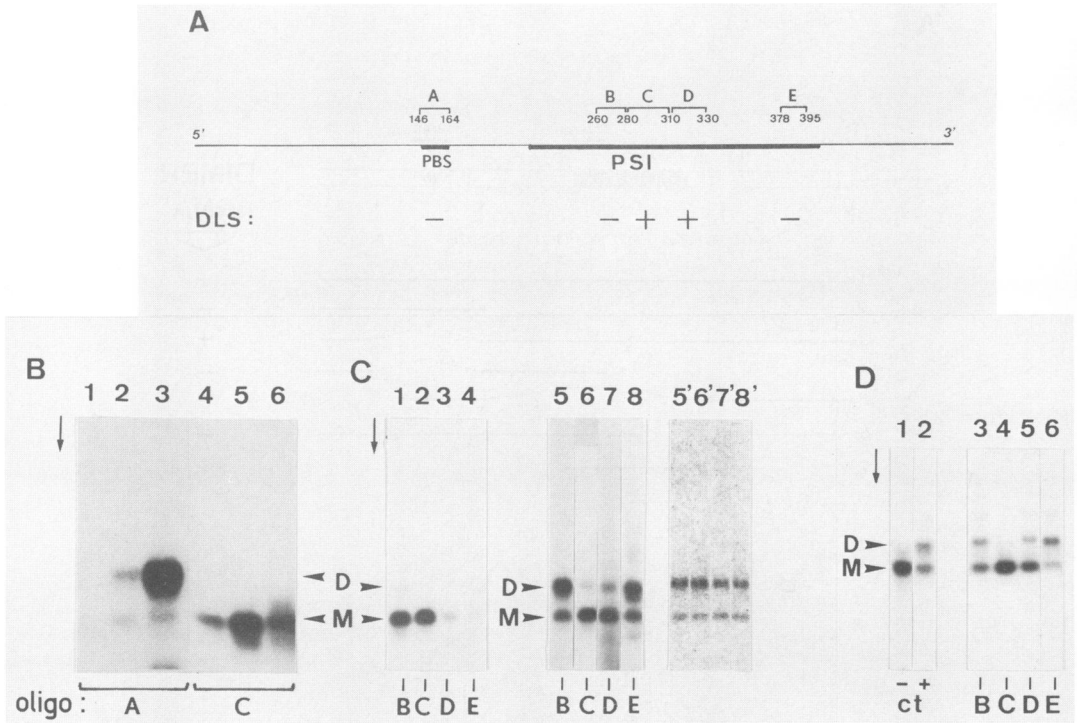

FIG. 5. Probing of the DLS by annealing DNA oligomers to MoMuLV RNA. (A) MuLV leader sequence from 5′ end to position 450 is drawn. *Psi* (positions 215 to 400; see references 21 and 33) and PBS (positions 146 to 164) are underlined. The five synthetic DNA oligomers (A through E) used for hybridization are A, B, C, D, and E and are complementary to RNA, as indicated (see Materials and Methods). The likely involvement of different parts of the leader sequence in the DLS, according to the hybridization results presented in Fig. 5B and 5C, is indicated by + or −. (B) Incubation of the MoMuLV 0.8-kb RNA was carried out in dimerization assay conditions (see Materials and Methods), with NCp10 and 50,000 cpm of 5′ [^{32}P]DNA oligomer A or C (Fig. 5A). Oligo-RNA ratio was 1:25. Electrophoresis and autoradiography were as described in Materials and Methods. Lanes 1 through 3, Oligomer A. Lane 1, control; lane 2, 20 ng of NCp10; lane 3, 80 ng of NCp10. Lanes 4 through 6, Oligomer C. Lane 4, control; lane 5, 10 ng of NCp10; lane 6, 40 ng of NCp10. M and D indicate monomer and dimer RNA positions detected upon ethidium bromide staining. The arrow indicates the direction of electrophoresis. (C) Incubations were performed as described in the legend to Fig. 5B, with DNA oligomers B, C, D, and E. Lanes 1 through 4, Controls without NC and an oligomer-RNA ratio of 1:25. Lane 1, Oligo B; lane 2, oligo C; lane 3, oligo D; lane 4, oligo E. Lanes 5 through 8, Incubations with 20 ng of RSV-NCp12, resulting in 60% RNA dimerization. The oligo-RNA ratio was increased to 1:1 for oligomers D and E to obtain a similar annealing level for all oligomers. Lane 5, Oligo B; lane 6, oligo C; lane 7, oligo D; lane 8, oligo E. Lanes 5′ through 8′ correspond to the ethidium bromide staining of lanes 5 through 8. (D) Oligonucleotides used to block RNA dimerization. Dimerization assays were performed in the presence of NCp10, as in Fig. 5B, with 150 ng of 0.22-kb pCR2 RNA labeled at 3×10^5 cpm/μg with [α-^{32}P]UTP (see Materials and Methods). Unlabeled oligonucleotides B, C, D, or E (1 μg) were added at the beginning of the dimerization reaction. After deproteinization, the level of RNA dimerization was analyzed by electrophoresis in an 1.2% agarose gel that was dried and autoradiographed as described in Materials and Methods. Lane 1, Control RNA alone; lane 2, plus NCp10 without oligonucleotide; lane 3, plus oligo B; lane 4, plus oligo C; lane 5, plus oligo D; lane 6, plus oligo E.

MuLV [24, 38; Friedrich, personal communication]), in the Rasheed rat sarcoma virus, in the feline leukemia virus and feline sarcoma virus, and in the simian sarcoma virus (38). Interestingly, mutations observed in the double-hairpin structure are compensated so that the structure is maintained (Fig. 7).

How two of these RNA sequences interact to maintain the DLS is still a matter of speculation. Palindromic sequences present at positions 280 to 330 are probably implied in intermolecular base pairings. However, the apparent 5′ to 5′ linkage holding the two RNA molecules cannot be explained by secondary structure only. Hence, the presence of tertiary RNA structures like pseudoknots may be suspected (39). Moreover, other hairpin structures are present in and outside the leader sequence, but they are not involved in the DLS (2, 28). This observation suggests that sequences surrounding the DLS sequence might be important to promote RNA dimerization. The results obtained from deletion mutagenesis in the MuLV leader sequence (Fig. 4) indicate that the *Psi* element (even shortened to 215 to 421) can promote RNA dimerization by itself, whereas sequences between positions 215 and 312 are necessary, but probably not sufficient (see Fig. 3, oligomer D). Clearly more work is needed to fully characterize the dimer promoting sequences.

The ability of MuLV RNA to dimerize in vitro and to form a DLS similar to that of MoMuLV 70S RNA (Fig. 2) (28) may seem unexpected, since previous attempts to reconstitute dimers with purified retroviral RNA were unsuccessful (15, 18, 28). However the use of in vitro transcription provided large quantities of intact RNA restricted to all or part of the 5′ 1,600 nucleotides of MoMuLV. This enabled us to use a high concentration of MoMuLV RNA in the dimerization assays and to focus attention on the dimer linkage structure and the dimerization process. Nevertheless, the inability of genomic RNA to spontaneously dimerize in vitro is probably the consequence of many RNA structures present along the RNA molecule (15, 18) as well as 5′-3′ end interactions (6) that must interfere with the dimerization process. These observations favor the idea that, in vivo, retroviral RNA dimerization must be catalyzed

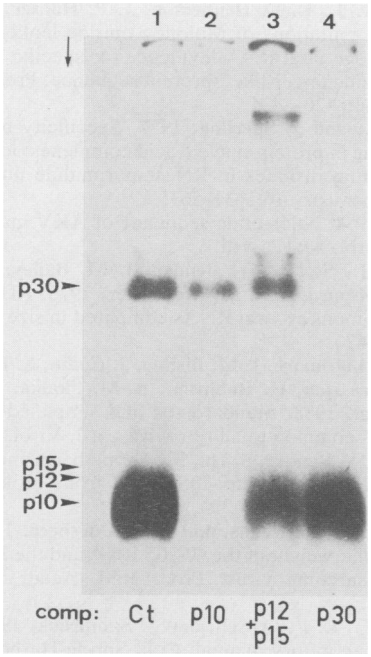

FIG. 6. Nucleocapsid protein NCp10 is bound to dimeric RNA in MuLV virions. UV light cross-linking of MuLV virions was carried out as described in Materials and Methods. The RNA-protein complexes obtained after T1 RNase digestion were immunoprecipitated with anti-MoMuLV protein A-Sepharose (19), and 5′ RNA was labeled with ^{32}P (see Materials and Methods). The cross-linked proteins were analyzed by SDS-PAGE, followed by autoradiography. To characterize the viral protein linked to the MoMuLV genomic RNA, each of the purified *gag* proteins was added as a competitor before immunoprecipitation with anti-MoMuLV serum (as indicated under each lane). Lane 1, No addition (control); lane 2, plus 1 μg of NCp10; lane 3, plus 1 μg of MAp15 and pp12; lane 4, plus 1 μg of CAp30. Arrows point to the positions of the four MuLV *gag*-coded proteins, as revealed by Coomassie blue staining of an identical gel.

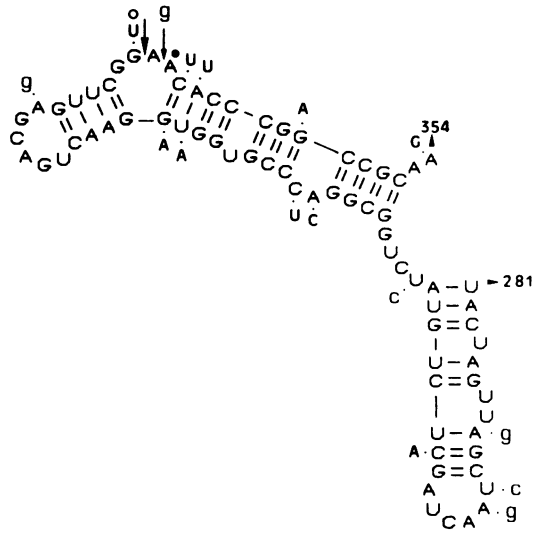

FIG. 7. Putative nucleotide sequence involved in the DLS of MoMuLV RNA. The secondary structure of the complete MoMuLV RNA leader was analyzed by T1 RNase protection assays (see Materials and Methods), and the data obtained were used to fold the MoMuLV RNA leader sequence (42). In the computed secondary structure of MoMuLV leader (A. C. Prats, Ph.D. thesis, Université Paul Sabatier, Toulouse, France, 1988), the sequence at positions 281 to 354 formed an independent domain with two hairpins. Computed structures of leader sequences from other MuLV species (AKV, Moloney, Friend, and radiation MuLV) indicated that this double hairpin structure was conserved (Prats and Darlix, unpublished results). The MoMuLV double hairpin structure is presented here. The large arrow indicates guanine residues susceptible to T1 RNase. Additional residues correspond to mutations seen in AKV, Moloney murine sarcoma virus, and spleen focus-forming virus. The black dot indicates deletion of an adenine residue. Residues in small letters correspond to mutations seen in Friend MuLV. The g with an arrow is an insertion, and the open dot indicates deletion of a guanine residue. This double hairpin structure has also been observed in 15 strains of murine, feline, and simian retroviruses. The estimated energy varies from −35 Kcal (MoMuLV) to −27 Kcal (simian sarcoma virus).

by the nucleocapsid protein or its precursor (*gag* polyprotein) or both, leading to dimeric RNA molecules further condensed to generate the 70S RNA found in mature virions.

NC protein appears to be, among several retroviral and nucleic acid-binding proteins tested, the only one able to catalyze RNA dimerization in vitro (31). NC protein could act by unwinding the RNA structure at and around positions 280 to 330, thereby accelerating interstrand base pairing interactions. However, other nucleic acid unwinding proteins like protein gp32 of T4 bacteriophage are unable to dimerize MoMuLV RNA (31). How can such an apparently nonspecific RNA-binding protein (32) cause MuLV RNA dimerization? The possible involvement of RNA sequences specifically recognized by NC protein is suspected, and this is presently under investigation.

Taking account of our results and of previous ones on RNA encapsidation, what might be the functional significance of MoMuLV RNA dimerization? The development of MuLV helper cell lines is based on the observation that a *cis*-acting element, named *Psi* and located at positions 215 to 565, is necessary for genomic RNA encapsidation into MoMuLV virions (20, 21). Furthermore other data have indicated that the element required for encapsidation is located between positions 215 and 400 (33), and here we show that the dimer promoting sequence is probably localized within the shortened *Psi* element. Also, recent genetic studies of NC protein have demonstrated that NCp10 is required for the packaging of MoMuLV genomic RNA (12, 23), and our biochemical analyses indicate that NC protein catalyzes MoMuLV RNA dimerization in vitro. Hence dimerization and encapsidation seem to depend on the same *cis* element and *trans*-acting factor, suggesting that the two processes might be linked. In disagreement with this hypothesis, it has been reported that RNA dimer formation takes place extracellularly during virion maturation (4). However this conclusion is based on data of sedimentation analyses of viral genomic RNA (60 to 70S for the dimer and 30 to 35S for the monomer) which could have been overinterpreted. In fact, the sedimentation of the genomic RNA dimer can vary from 45 to 70S depending on its state of condensation (1), and, furthermore, the immature RNA dimer appears to be unstable during its purification unless 0.5 M NaCl is added (36). Last but not least, 70S RNA has been identified both in RSV- and MoMuLV-infected cells (9; Gabus and Darlix, unpublished results). In conclusion, these observations favor the idea that dimerization and encapsidation of MuLV RNA could be linked processes controlled by NC protein. Presently we speculate that formation of the DLS catalyzed by NC protein or *gag* precursor molecules or both could be the first step toward the encapsidation of the genomic RNA.

In the course of virion budding and maturation, several other interstrand interactions should be catalyzed by NC protein molecules, leading to a condensed 70S RNA (1, 5, 7, 18). In agreement with this hypothesis, we show that NCp10 molecules are tightly bound to the genomic RNA in MoMuLV virions (Fig. 6). If, however, RNA dimerization occurs only upon virion maturation, then its role might be restricted to the interstrand DNA transfer during reverse transcription (29, 40). Finally, the association between NC protein molecules and dimeric RNA in the viral particle may well form a circular chromatin-like structure, as has been observed for avian myeloblastosis virus (6), which then could serve to facilitate the synthesis of a complete proviral DNA molecule.

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